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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Through the application of SAGE and array technologies, our laboratory has identified genes, such as Claudin-7, that are lost in metastatic breast cancer cells. We plan to apply magnetic resonance imaging and spectroscopy to determine the impact of Claudin-7 on breast cancer cell invasion and metabolism. Breast cancer cells engineered to stably overexpress Claudin-7 will be generated. MR microscopy methods will be used to quantify invasion of cells into Matrigel and localized 1H MRS and 31P MRS will be used to study the physiology and metabolism of cells during invasion. Alterations in tight junction characteristics will be analyzed to study correlation to the acquired phenotypes. To characterize vasculature in terms of vascular volume and permeability using high resolution MRI, tumor metabolic characteristics, namely, lactate levels, intra- and extra-cellular pH, and phospholipid metabolism will be obtained by multi-nuclear (1H and 31P) spectroscopic imaging of solid tumors. These aims will be performed using the following human breast cancer cell lines: non-metastatic cell lines MCF-7 and SKBR3, and the metastatic cell line, MDA-MB-435.

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Table of Contents

Background:

Approximately 30 percent of node-negative and 75% of node-positive patients with breast cancer experience a relapse and die from their disease within 10 years. Survival rates for metastatic breast cancer remain pitifully low and the challenge of our decade is to find ways to prevent cancer cells from disseminating. Divested of this aspect, breast cancer ceases to be life threatening.

Several novel genetic alterations have recently been identified in invasive metastatic breast cancer, in our laboratory, by SAGE and array analysis [1, 2, 3]. One striking alteration is the 10- to 100-fold overexpression of Claudin-7 in metastatic tumors compared to normal breast epithelium [1]. Claudin-7 is a newly cloned member of a family of proteins, typified by four transmembrane domains, which are components of tight junctions (TJ) of epithelial cells [4, 5].

Tight junctions and their role in cancer and metastasis.

TJs occur at the most apical part of lateral membranes of simple epithelial cells, and serve as a permeability barrier, and restrict the lateral diffusion of membrane lipids and proteins between the apical and basolateral compartments to maintain cell polarity. Events commonly associated with the oncogenic transformation of epithelial cells include the loss of cell-cell contacts and the acquisition of more migratory and invasive phenotypes [6]. Thus, tumor cells capable of metastasis appear to have loose TJs, while cells that do not metastasize have tight connections or junctions between them [7, 8], leading to the speculation that the nature of these connecting strands can determine the efficiency with which tumor cells break away from each other and disseminate. Moreover, introduction of exogenous occludin reversed the process, and resulted in a reacquisition of normal epithelial monolayer phenotype and functionally intact TJs [9]. These studies provide strong evidence for the importance of structurally and functionally intact TJs in epithelial cells, and their disruption in cancer.

As a first step towards this goal, we needed to identify genes that confer the metastatic phenotype to breast cancer cells. Through the application of SAGE and array technologies, our laboratory has identified genes, such as Claudin-7, that are differentially expressed in primary versus metastatic breast cancer [1].

As a second step, we need to apply reliable methods to study invasion, tumor metabolism and vascularization, preferentially *in vivo*. Magnetic resonance imaging and spectroscopy are emerging technologies that could fulfil these requirements.

A major shift in our thought process has taken place in this last one year, based upon our new findings regarding Claudin-7. In 1999, Claudin 7 was found to be overexpressed in 50% of primary breast cancers in array and SAGE analysis when compared to gene expression in finite lifespan mammary epithelial cells placed in short term culture, obtained from Clonetics. Further characterization of Claudin 7 expression by quantitative real time PCR, immunohistochemistry and Western analysis in tumor sections showed that, contrary to expectation, Cl-7 expression is lost, not gained as originally reported. Tumro cells most often had lost Cl-7 expression, while normal adjacent ducts expressed abundant Cl-7. Thus, we had been misled in our SAGE and array analysis by using primary normal cells; but culturing these cells had abolished Cl-7 expression in them. Use of these cells is rampant in the scientific community. For most genes, it does seem to make any difference, but for Claudin-7, which forms tight junctions between cells, it was very important. Compared to these cells, of course, tumors appeared to over-express the gene. In fact, based on solid new evidence (see appended paper), it is clear that 50% of tumors lose expression of the gene.

Our hypothesis and specific aims remain the same, although now we will try to see whether Claudin-7 normally behaves like a tumor suppressor gene, so that loss of the gene allows breakdown of the junctional connections and permits cells to disperse and disseminate.

Hypothesis: Breast cancer cells genetically engineered to overexpress the tight junction protein, Claudin-7, will exhibit significant differences in invasion, vascularization, metabolism and metastasis compared to the non-metastatic breast cancer cells.

Objectives:
Aim 1. (Cell studies): To determine the impact of Claudin-7 on breast cancer cell invasion and metabolism. Breast cancer cells engineered to stably overexpress Claudin-7 will be generated. MR microscopy methods will be used to quantify invasion of cells into Matrigel and localized 1H MRS and 31P MRS will be used to study the physiology and metabolism of cells during invasion. Alterations in tight junction characteristics will be analyzed to study correlation to the acquired phenotypes.

Aim 2. (In vivo studies): To characterize vasculature in terms of vascular volume and permeability using high resolution MRI. Solid tumors derived from the breast cancer cell lines will be studied in vivo. Tumor metabolic characteristics, namely, lactate levels, intra- and extra-cellular pH, and phospholipid metabolism will be obtained by multi-nuclear (1H and 31P) spectroscopic imaging of solid tumors. These aims will be performed using the following human breast cancer cell lines: non-metastatic cell lines MCF-7 and SKBR3, and the metastatic cell line, MDA-MB-435.

Statement of Work and accomplishments, 2001-2002

Task 1. (months 1-6). Generate clones of Claudin-7, tagged and untagged with marker genes, transfect cells, obtain stable clones in all three cell lines, MCF-7, SKBR3, and MDA-MB-435. Confirm the increased expression of Claudin-7 in transgenic cells and solid tumors derived from transgenic cells.

a. we generated a peptide-specific polyclonal antibody against Cl-7. This antibody was characterized and applied to Western and immunohistochemical analysis. The antibody was specific and provided excellent evidence for Cl-7 loss of expression in primary breast cancers.
b. Quantitative RT-PCR analysis was performed on 10 breast cancer cell lines and 10 primary epithelial cells obtained immediately after reduction mammoplasty. It was confirmed that normal mammary epithelial cells express easily detectable levels of Claudin-7, while most tumor cells do not.
c. Transfection of Cl-7 into MDAMB435 and MDAMB231, two metastatic cell lines and HBL100, a normal HMEC were attempted. HBL100 yielded many stable clones, while clones of MDAMB231 express mRNA but no protein. This curious phenomenon could be because of a unique Cl-7 protein breakdown system in these cells. But without stable expression of protein, these cells will not be amenable to further analysis. We have found that MCF-7 cells express abundant Cl-7, but its adriamycin- resistant counterpart does not express any detectable Cl-7. We plan to derive stable clones of MCF-7ADR cells, and use the pair to perform mouse experiments and task 2.
d. We have yet to perform the animal experiments.

Task 2: (months 3-12): Study the TER, enzymatic dispersion and reaggregation characteristics of Claudin-7 transfectants compared to parental cells.

Not initiated yet because of the difficulties in obtaining stable clones from breast cancer cell lines. We will be able to complete this in the course of the next 2-3 months, after which we will embark upon Task 2.

Task 3. (months 5-17). Characterize invasive behavior and metabolic profiles of control and transgenic cells for MCF-7, SKBR3 and MDA-MB-435 cells.

At least 5 separate experiments will be performed to establish significant differences for transgene and control cells. The cell studies will be performed using a multi-nuclear probe. Therefore, 31P and 1H spectra together with invasion data will be obtained during the same experiment.

Task 4. Characterize vascular and metabolic characteristics of control and claudin-7 transgenic tumors derived from the cell lines. The in vivo experiments will require separate groups of animals and these are outlined below.

(months 11-18) MCF-7 tumors:
(n=15, control vector, 31P MR studies; n=15, CLAUDIN-7 transgene tumors, 31P MRS studies)
(n=15, control vector, 1H MRS studies; n=15, CLAUDIN-7 transgene tumors, 1H MRS studies)
(n=15, control vector, vascular characterization; n=15, CLAUDIN-7 transgene tumors, vascular characterization)

(months 18-24) MDA-MB-435 tumors.
(n=15, control vector, 31P MR studies; n=15, CLAUDIN-7 transgene tumors, 31P MRS studies)
(n=15, control vector, 1H MRS studies; n=15, CLAUDIN-7 transgene tumors, 1H MRS studies)
(n=15, control vector, vascular characterization; n=15, CLAUDIN-7 transgene tumors, vascular characterization)

(months 25-31) SKBR3 tumors.
(n=15, control vector, 31P MR studies; n=15, CLAUDIN-7 transgene tumors, 31P MRS studies)
(n=15, control vector, 1H MRS studies; n=15, CLAUDIN-7 transgene tumors, 1H MRS studies)
(n=15, control vector, vascular characterization; n=15, CLAUDIN-7 transgene tumors, vascular characterization)

Task 5: (months 31-36) Complete experiments. Repeat any tissue culture or animal experiments needed. Analyze data, write up manuscripts and reports, and present data.

Key research accomplishments: see appended paper.

Reportable outcomes:

Manuscript: Scott L. Kominsky, Pedram Argani, Dorian Korz, Ella Evron, Venu Raman, Elizabeth Garrett, Alan Rein, Guido Sauter, Olli-P Kallioniemi, and Saraswati Sukumar Loss of the Tight Junction Protein Claudin-7 Correlates With Histological Grade In Both Ductal Carcinoma In Situ and Invasive Ductal Carcinoma of the Breast (submitted to Cancer Res, May 2002)

Presentations:
Breast Spore meeting at Dana Farber Cancer Center, Boston, MA, October 2001
Breast Cancer Research Meetings, - December. 10-13, 2001, San Antonio, TX -
10th SPORE Investigators' Workshop –Early detection of breast cancer cells in ductal lavage fluid – quantitative assessment of cyclin D2, RAR-β, Twist, RASSF1A, and HIN-1 by real time methylation specific PCR (abstract). July 13-16, 2002.
Annual Meeting of Society of Gynecology and Obstretics, April 3-5, Innsbruck, Austria
Association of Investigative Pathologists, April 20, 2002
American Radium Society Meeting – April 27-30, 2002, Las Croabas, Puerto Rico
CYTYC Health Corporation – May 23, 2002, Boxborough, MA.
Abbott Laboratories – May 10, 2002, Abbott Park, IL

Patent application – Aberrantly Methylated Genes as Markers of Breast Malignancy (Docket # JHU1630; Ref. # DM-3729)

Conclusions:

Claudins are transmembrane proteins that seal tight junctions, and are critical for maintaining cell-to-cell adhesion in epithelial cell sheets. However, their role in cancer progression remains largely unexplored. We have reported that Claudin-7 expression is lost in invasive ductal carcinomas (IDC) of the breast, as determined by both RT-PCR (9 of 10) and Western analysis (6 of 8). Immunohistochemical (IHC) analysis of ductal carcinoma in situ (DCIS) and IDC showed that the loss of Claudin-7 expression correlated with histological grade in both DCIS ($p < 0.001$, $n = 38$) and IDC ($p = 0.014$, $n = 31$), occurring predominantly in high-grade (Nuclear and Elston grade 3) lesions. Tissue microarray analysis of 355 IDC cases further confirmed the inverse correlation between Claudin-7 expression and histological grade ($p = 0.03$). This pattern of expression is consistent with the biological function of Claudin-7, as greater discohesion is typically observed in high-grade lesions. In line with this observation, by IHC analysis, Claudin-7 expression was lost in the vast majority (13/17) of cases of lobular carcinoma in situ, which is defined by cellular discohesion. In fact, inducing discohesion of MCF-7 cells in culture by treating with HGF/scatter factor resulted in a rapid loss of Claudin-7 expression. In breast cancer cell lines, our data suggests that silencing of Claudin-7 expression occurs by promoter hypermethylation. In summary, these studies provide insight into the potential role of Claudin-7 in the progression and ability of breast cancer cells to disseminate, and suggest that Claudin-7 may be valuable as a prognostic indicator for breast cancer.

Future work will address the dissection of the role of Claudin 7 in invasion and metastasis using cell culture, animal model and imaging systems.

The proposed studies will identify physiological and metabolic alterations associated with Claudin-7. A new role for a little known class of proteins, which are components of the tight junctions, will be established. Such physiological and metabolic characterizations will provide rationales for therapeutic designs to prevent metastasis, as well as provide MR detectable risk factors for evaluating disease aggressiveness.

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Appendices:

Scott L. Kominsky, Pedram Argani, Dorian Korz, Ella Evron, Venu Raman, Elizabeth Garrett, Alan Rein, Guido Sauter, Olli-P Kallioniemi, and Saraswati Sukumar Loss of the Tight Junction Protein Claudin-7 Correlates With Histological Grade In Both Ductal Carcinoma In Situ and Invasive Ductal Carcinoma of the Breast (submitted to Cancer Res, May 2002)